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Functional analysis of virion host shutoff protein of pseudorabies virus

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Abstract

During lytic infection, the virion host shutoff (vhs) protein of alphaherpesviruses causes the degradation of mRNAs nonspecifically. In this work, we cloned the vhs gene (UL41 open reading frame) of pseudorabies virus (PRV; TNL strain) by PCR, and its nucleotide sequences were determined. The PCR product of vhs gene was subcloned into the prokaryotic pET32b expression vector, and production of the recombinant vhs protein was examined by SDS-PAGE. Result of Western blotting demonstrated that our recombinant vhs protein reacted with antiserum against a synthetic peptide of 17 amino acids of the vhs protein. After purification with nickel-chelate affinity chromatography, the purified recombinant vhs protein exhibited in vitro ribonuclease activity as expected. We further cloned the vhs gene into eukaryotic expression vectors and investigated the intracellular function of vhs protein by DNA transfection. By transient trasfection and CAT assay, we found the CAT activity was reduced in the presence of vhs, indicating that degradation of mRNA of the CAT gene was caused by the vhs. Furthermore, our results showed that the plaque formation of pseudorabies virus was blocked by exogenous vhs. Taken together, we have cloned the vhs gene of pseudorabies virus (TNL strain) and conducted functional analysis of the recombinant vhs protein in vitro as well as in vivo.

Keywords: Pseudorabies virus; vhs gene

Introduction

Pseudorabies virus (PRV) is a member of *Alphaherpesvirinae* in the family *Herpesviridae*, causing Aujeszky's disease of pigs (Mettenleiter, 1994). Human herpes simplex virus 1 (HSV-1) is the prototype of this family and has been extensively studied (Roizman and Knippe, 2001). HSV-1 carries a structural protein, the virion host shutoff (vhs) protein that causes the inhibition of host protein synthesis by degradation of cellular mRNAs after infection (Fenwick and Clark, 1982). The vhs protein is encoded by the UL41 gene and is a component in the tegument of a virion. Upon viruses entering into the cell, this tegument protein exerts its deleterious effects to the host immediately and does not require virus protein synthesis (Becker et al., 1993; Karr and Read, 1999; Oroskar and Read, 1989; Read, 1997; Read et al., 1993; Schek and Bachenheimer, 1985; Smibert et al., 1992). Following the onset of viral gene expression, vhs also causes the decay of viral mRNAs, implying that vhs plays a role in the regulation of differential expression of viral genes (Fenwick and Everett, 1990; Kwong and Frenkel, 1987; Oroskar and Read, 1989; Read, 1997). In late time of lytic infection, the vhs protein is produced again, sequestered and inactive, and eventually packaged into the progeny virions (Kwong and Frenkel, 1987, 1989; Lam et al., 1996; Read et al., 1993; Smibert et al., 1994).

The precise mechanism of accelerated mRNA turnover and inhibition of protein synthesis remains unclear. The requirement of mammalian factor(s) for efficient activity of vhs protein has been suggested (Lu et al., 2001). In addition, it was found that the vhs is a viral mRNA degradation factor, targeted to mRNAs and to the translation initiation stage, through an interaction with initiation factor eIF4H (Feng et al., 2001).

The UL41 gene is conserved in alphaherpesviruses, including HSV-1, HSV-2, PRV, varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV-1), and equine herpesvirus 1 (EHV-1) (Davison and Scott, 1986; Everett and Fenwick, 1990; Feng et al., 1996; Hinkley et al., 2000; Telford et al., 1992). An unusual feature of the vhs of alphaherpesviruses is

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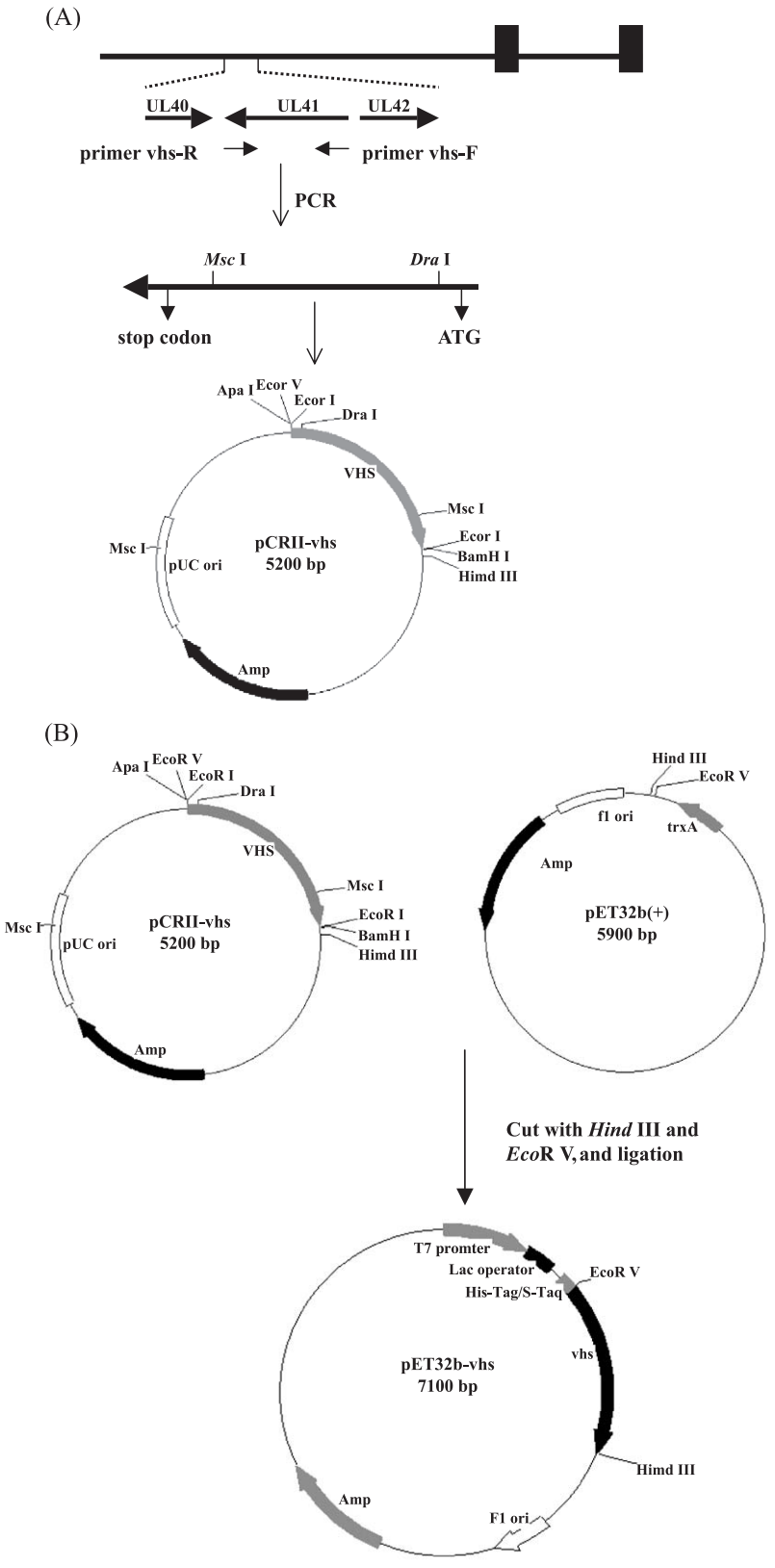


Fig. 1. Diagram of cloning of PRV vhs gene. (A) The vhs gene is in the UL41 region of PRV genome. The vhs-R and vhs-F were primers used in the PCR. The DNA fragment (approximately 1.2 kb) of PCR was subcloned into the pCRII vector and the resulting plasmid was named pCRII-vhs. (B) Construction of prokaryotic vector expressing vhs protein. The pCRII-vhs was cut with *Hind*III and *Eco*RV to generate the insert, which was further cloned into the corresponding enzyme sites of pET32b. The prokaryotic expressing plasmid was named as pET32b-vhs.

that it lacks primary sequence similarity to known RNases except a weak similarity to the FEN-1family of nucleases (Everly et al., 2002; Lu et al., 2001; Stevens, 1998). Although the sequences of the vhs gene in PRV genome have been documented (Berthomme et al., 1993), the biochemical properties of the PRV vhs gene product have not been studied yet. In this paper, we reported the cloning of the vhs gene of PRV and functional studies on the recombinant vhs protein.

Results and discussion

The vhs gene is in the UL41 region of PRV genome (Berthomme et al., 1993). Using appropriate primers (vhs-R and vhs-F) and applying PCR, we have successfully cloned the vhs gene. The DNA fragment (approximately 1.2 kb) of PCR was further subcloned into the pCRII vector (Fig. 1) and the resulting plasmid was designated as pCRII-vhs. Automated DNA sequenceing was carried out to confirm the nucleotide identity of our PCR product. Our sequence data of vhs gene of TNL strain exhibited a 97.8% similarity to that of Ka strain; the deduced amino acid sequence similarity was 98%.

To express the recombinant vhs protein, the pCRII-vhs was cut with *Hin*dIII and *Eco*RV to generate the insert, which was further cloned into the pET32b prokaryotic expressing vector. The expressing plasmid was named as pET32b-vhs (Fig. 1).

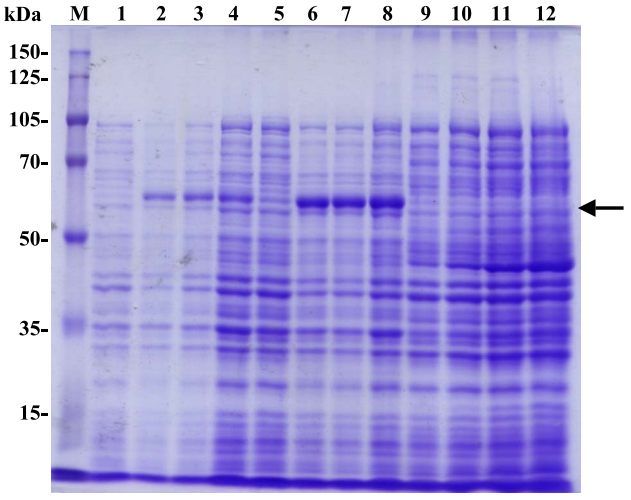


Fig. 2. Expression of recombinant vhs protein in *E. coli*. The pET32b-vhs plasmid was introduced into *E. coli* BL21 (DE3) pLysS and the expression of vhs was under the control of IPTG induction. The lysate was separated by SDS–10% polyacrylamide gel electrophoresis, and the gel was stained with Coomassie blue. M, protein molecular mass markers. Lanes 1–4: expression of recombinant vhs protein (pET32b-vhs, colony 1). Lanes 5–8: expression of vhs protein (pET32b-vhs, colony 2). Lanes 9–12: pET32b as negative control. Lanes 1, 5, and 9 were without IPTG treatment. All other lanes were treated with IPTG (1, 3, 5 h, respectively, from left to right). The arrowhead indicates the position of recombinant vhs protein (59 kDa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

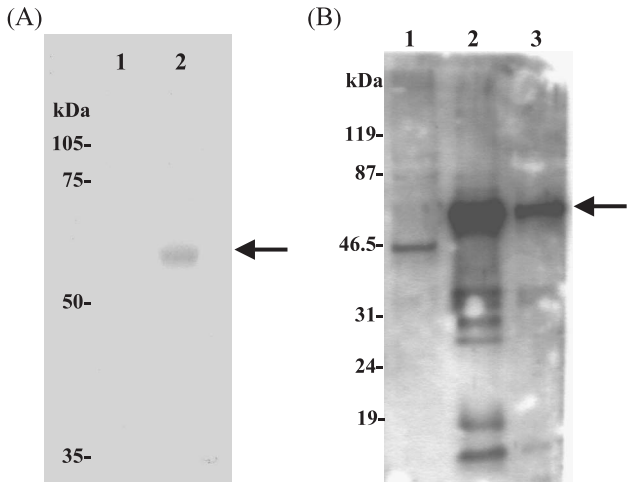


Fig. 3. Purification and identification of vhs protein. The recombinant protein was purified by affinity chromatography, analyzed by SDS-PAGE, and identified by Western blotting. (A) Lane 1: pET32b (IPTG induction 5 h) as negative control. Lane 2: pET32b-vhs with IPTG induction. The antibody was rabbit polyclonal anti-PRV antibody. (B) Lane 1: sample of PRV virion preparation from sucrose gradient. Lanes 2 and 3: purified recombinant products eluted with buffer containing 250 or 200 mM imidazole, respectively. The native vhs protein in the virion (lane 1) was smaller than the recombinant fusion protein, which contained an extra peptide (Trx • Tag) at N-terminus (lanes 2 and 3). The antibody was rabbit polyclonal antibody against a synthetic peptide of vhs as described in Materials and methods. The arrowheads indicate the position of recombinant vhs protein.

The pET32b-vhs was introduced into *E. coli* BL21 (DE3) pLysS and IPTG was used for the induction of the vhs protein. As shown in Fig. 2, the results of SDS-PAGE demonstrated that significant amounts of the recombinant vhs protein (59 kDa) were produced. The partial identity of this recombinant protein was examined by N-terminal amino acid sequencing to ensure the reading frame is correct (data not shown).

Although the biochemical and biological functions of the vhs gene of HSV were investigated extensively, it appears that the high level of expression and purification of a recombinant vhs protein in prokaryotic system was not reported yet (Elgadi et al., 1999; Everly et al., 2002; Krikorian and Read, 1991; Strelow and Leib, 1995, 1996). In the following, we performed several in vitro and in vivo experiments to examine the properties and functions of the recombinant vhs protein of PRV.

For immunological study, the recombinant vhs protein was purified by affinity chromatography, analyzed by SDS-PAGE, and identified by Western blotting. We found that it could be recognized either by rabbit polyclonal anti-PRV antibody or by rabbit polyclonal antibody against a synthetic 17-amino-acid peptide of vhs (Fig. 3). The native vhs protein of the PRV virion could also react with the polyclonal antibody against a synthetic peptide of vhs (Fig. 3B). Our Western blot data revealed that this 17-amino-acid peptide (between box II and box III of the vhs polypeptide) is an epitope shared by the recombinant vhs and native vhs protein in the virion. There are four conserved regions

(boxes I, II, III, and IV) in the vhs genes of herpesviruses (Berthomme et al., 1993).

To examine the in vitro RNase activity of recombinant vhs protein, RNA markers were incubated with purified vhs protein and analyzed with agarose gel electrophoresis. Results showed that the purified vhs protein degraded RNA samples rapidly (Fig. 4A, lanes 4–6). By contrast, the negative control experiment, in which the reaction solution was eluted from empty vector pET32b, demonstrated that no endogenous ribonuclease activity was observed (Fig. 4A, lanes 1–3), indicating that the RNA was degraded by the recombinant vhs protein. We also constructed a vhs mutant with four-amino-acid (SDRG) deletion in the conserved box II region and we found this mutant demonstrated partial nuclease activity (Fig. 4B). This observation was consistent with the in vitro ribonucleolytic activity of HSV-1 vhs protein, which was extracted from virions or was in vitro-translated (Zelus et al., 1996). A recent report also provided the evidence that the vhs protein of HSV-1 is an RNase, either alone or forming a complex with translational factor eIF4H (Everly et al., 2002). Moreover, we tested the DNase activity of purified vhs protein, and we found no DNase activity was associated with the vhs protein preparation (data not shown).

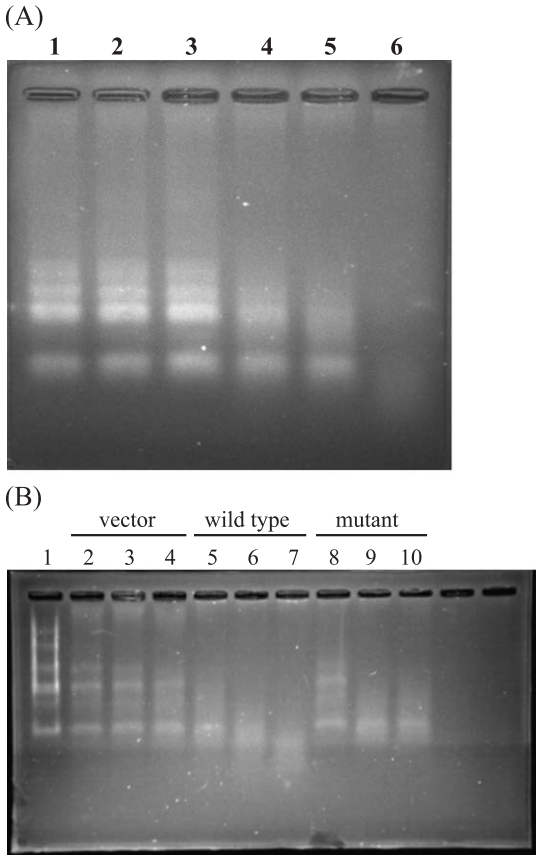


Fig. 4. (A) Assay of in vitro RNase activity of purified recombinant vhs. RNA marker was incubated with purified vhs protein and analyzed with agarose gel electrophoresis. Lanes 1–3: negative control. RNA was incubated with solution containing Trx•Tag of pET32b (20, 40, and 80 min, respectively). Lanes 4–6: RNA marker was incubated with vhs (20, 40, and 80 min, respectively). The RNA samples were almost degraded completely by the vhs (lanes 4–6). (B) Mutational study of vhs protein. Lane 1 is RNA markers. Lanes 2–4: incubation of RNA substrate with eluted solution using empty vector in transforming *E. coli*. The reaction time was 20, 40, and 80 min, respectively, from lanes 2 to 4 and the reaction temperature was 37 °C. A small portion of RNA degradation observed in lane 4 was probably due to the intrinsic instability of RNA. Lanes 5–7: incubation of RNA with purified wild-type vhs protein. The reaction condition was same as lanes 2–4. RNA was degraded obviously in the 20-min reaction (lane 5). Lanes 8–10: incubation of RNA with purified mutant vhs containing four-amino-acid (SDRG) deletion. The reaction condition was same as lanes 2–4. RNA was not degraded in the 20-min reaction (lane 8), but was degraded in the 40-min reaction (lane 9).

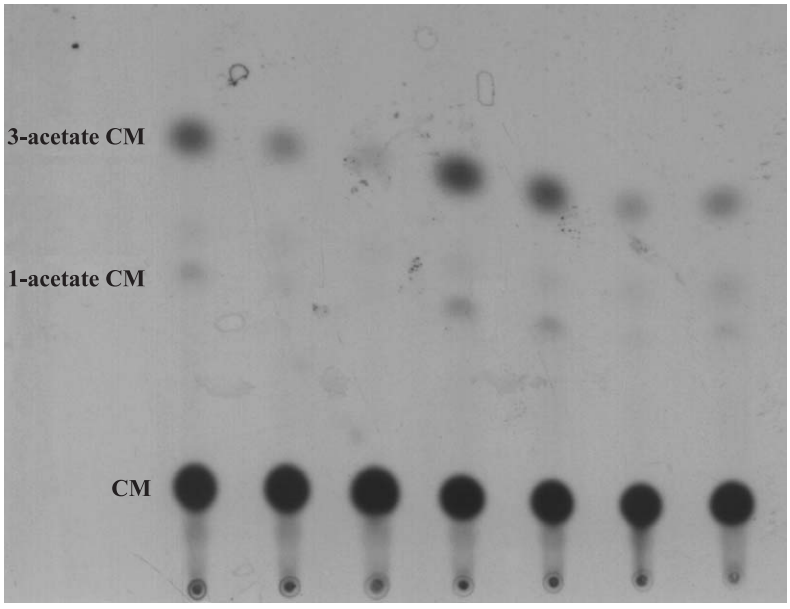
To assess the intracellular function of vhs gene, we cloned the vhs gene into eukaryotic expressing vectors. The expression of the vhs gene was under the control of a cytomegalovirus (CMV) promoter (pcDNA3.1-vhs) or a gE promoter (pgEp-vhs) of PRV glycoprotein E, which was cloned in our laboratory (Chang et al., 2002). Previous studies have shown that vhs of HSV can function in the absence of other viral gene products (Jones et al., 1995; Pak et al., 1995). Using transient transfection and CAT assay, we observed that the CAT activity was blocked by cotransfection of plasmid containing vhs gene (Fig. 5). It was reasonable to conclude that the reduced CAT activity resulted from the intracellular degradation of mRNA of CAT gene by vhs and that the vhs of PRV can also be functional in the absence of other viral products.

It was shown that the vhs protein of HSV inhibited the replication of human immunodeficiency virus (Hamouda et al., 1997). Therefore, we explored the effect of the recombinant vhs on the plaque formation of PRV. Cells were transfected with pgEp-vhs or pgEp-EGFP (EGFP, enhanced green fluorescent protein gene; Clontech, BD Biosciences); both plasmids bear the promoter of PRV glycoprotein E gene to control vhs or EGFP gene, respectively (Chang et al., 2002). Following 16 h of transfection, cells were infected with PRV; then plaque assay was conducted. We found that there was an approximately 70% reduction in plaque formation in the presence of vhs. In contrast, the result of a similar experiment using EGFP was essentially same as that of the control (Fig. 6). Our finding indicated that the vhs, through its nonspecific ribonucleolytic activity, caused global damage to the cell function and accordingly the following PRV infection was unable to finish normally.

Materials and methods

Virus and cells

The genomic DNA of pseudorabies virions (TNL strain) was isolated from PRV-infected Madin–Darby bovine kidney (MDBK) cells by sucrose gradient as described previously (Wong and Chen, 1998). LM (tk^-) cells were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL) supplemented with 10% fetal bovine serum



	1	2	3	4	5	6	7
pCAT-Promoter (3μg)	+	+	+	+	+	+	+
pcDNA3.1-vhs (μg)	1	3	6	-	-	-	-
pgEp-vhs (μg)	-	-	-	-	1	3	6
pcDNA3.1 (3μg)	-	-	-	+	-	-	-

Fig. 5. Inhibition of CAT activity by cotransfection of plasmid containing vhs gene. Cells were transfected with pCAT-Promoter or cotransfected with plasmids containing vhs (pcDNA3.1-vhs and pgEp-vhs). Here, the CAT activity of pCAT-Promoter (lane 4) was used as standard for examining the suppressive effect of the vhs gene that was driven either by a cytomegalovirus (CMV) promoter (pcDNA3.1-vhs) or by a gE promoter (pgEp-vhs) of PRV glycoprotein E. The results demonstrated that the CAT activity of pCAT-Promoter was inhibited by vhs (lanes 1–3 and lanes 5–7).

(Hyclone), penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Polymerase chain reaction (PCR) and sequencing

Genomic DNA of PRV was used as templates in PCR. PCR was carried out in a 50-μl mixture containing 1 μl (0.1 μg) viral DNA, 2 μM of the primers vhs-F (5′-TGTGCGAGCGGAGACATGGGCT-3′) and vhs-R (5′-AGAGGGCGAGCATCACAC-3′), 1.25 u of Taq DNA polymerase (TaKaRa), 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, and 5 μl DMSO. The PCR program was 35 cycles consisting of 1 min at 94 °C for denaturation, 30 s at 55 °C for annealing, and 1 min at 72 °C for extension. A DNA fragment of 1.2 kb was of the PCR product was examined by agarose gel electrophoresis, and the desired DNA fragments were eluted for the following experiment. The nucleotide identity of the PCR product was determined by automated DNA sequencer.

Plasmid construction

PCR products were cloned into the pCRII vector (Invitrogen), and the result plasmid was named pCRII-vhs. The pCRII-vhs was checked with restriction enzyme digestion and agarose gel electrophoresis. The DNA fragment of the vhs gene was cleaved out by *Eco*RV–*Hin*dIII and subcloned into the *Eco*RV–*Hin*dIII sites of pET32b vector (Novagen) and the resulting plasmid was named pET32b-vhs.

To construct plasmids for transfection experiments, the DNA fragment of the vhs gene was cleaved out from pCRII-vhs by *Eco*RV–*Bam*HI and subcloned into the *Eco*RV–*Bam*HI sites of pcDNA3.1 eukaryotic expression vector (Invitrogen), and the resulting plasmid was named as pcDNA3.1-vhs.

Mutant construction

Genomic DNA of PRV was used as template in PCR. PCR was carried out in a 50-μl mixture containing 1 μl (0.1 μg) viral DNA, 2 μM of the primers vhs-F (5′-TGTGCGAGCGGAGACATGGGCT-3′) and vhs-Rd-1(GCGGTCGTCGACGAAGATGGGGAAGTA-3′) or vhs-FdN (5′-GTCTCGGACCGCGATATCTTCGGGAAC-3′) and vhs-R (5′-AGAGGGCGAGCATCACAC-3′), 1.25 u of Taq DNA polymerase (TaKaRa), 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, and 5 μl DMSO. The PCR program was 45 cycles consisting of 30 s at 94 °C for denaturation, 30 s at 55 °C for annealing, and 1 min at 72 °C for extension. The nucleotide identity of the PCR product was determined by automated DNA sequencer.

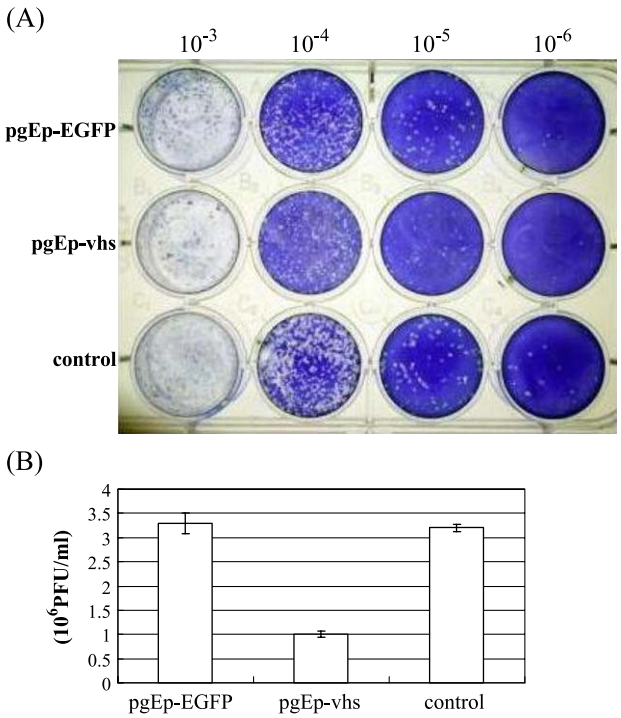


Fig. 6. Inhibition of plaque formation of PRV by vhs. (A) Cells were either mock or were transfected with pgEp-vhs or pgEp-EGFP. At the 16–18 h after transfection, cells were infected with PRV. The inoculum was removed and cells were then overlaid with medium containing methylcellulose. Plaques were visualized by staining with crystal violet and photographed. (B) Quantitation of plaque assay; each column shows the mean value of three independent experiments, and each vertical bar indicates the standard deviation. The vhs could cause an approximately 70% reduction in plaque formation; in contrast, result of the EGFP was essentially same as that of the control.

PCR products were cloned into the pGEM-T-Easy vector (Promega), and the result plasmids were named pGEM-T-Z-vhs-F/Rd-1 and pGEM-T-Z-vhs-Fd/R. The insert in pGEM-T-Z-vhs-F/Rd-1 was cleaved out by *Hin*cII and subcloned into the *Eco*RV site of pGEM-T-Z-vhs-Fd/R, and the resulting plasmid was named pGEM-T-Z-vhsm3, which was a vhs mutant with four-amino acid deletion. The DNA fragments of the wild-type vhs and mutated vhsm3 were cleaved out by *Pst*I and subcloned into the *Pst*I sites of pET44a expression vector (Novagen), respectively; and the resulting plasmids were named pET44a-vhs and pET44a-vhsm3.

Expression, purification, and identification of vhs protein

The pET32b-vhs was introduced into *E. coli* BL21 (DE3) pLysS and the expression of vhs was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) treatment. The bacterial lysate was separated by SDS–10% polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was stained with Coomassie blue. For purification of 6xHis-tagged protein under native condition, we used nickel-chelate affinity chromatography to purify the recombinant vhs as well as the control Trx•Tag protein (QIAGEN). For immunoblotting, the gel was further transferred to a nitrocellulose membrane (NEN). The membrane was incubated in blocking buffer for 1 h at room temperature and then incubated for 1 h with rabbit antiserum (CashmereBiotech, Taipei) against a synthetic peptide (amino acid one-letter symbol: ADGDGAADAPPRPRWST) of vhs protein at 800-fold dilution. Unbound antiserum was removed by washing the membrane three times in PBST for 5 min. The second antibody was peroxidase-labeled anti-rabbit antibody (NEN) and was incubated for 1 h at room temperature. The final method for detection was the ECL detection system (Amersham Pharmacia Biotech).

N-terminal amino acid sequencing

Following SDS-PAGE, the gel was stained with Coomassie blue and the proteins were transferred to PVDF membrane (NEN Life Science). The desired band on the membrane was cut off for automated amino acid sequencing, which was carried out in the Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan.

RNase activity in vitro

The commercial RNA marker (1 mg/ml; Ambion) (1 μg) and purified vhs protein (1 μg) were co-incubated in the elution buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole) at 37 °C for 20, 40, and 80 min, respectively. Then the reaction mixture was analyzed with 1% agarose RNA denaturing gel.

Transfections and CAT assay

Using the Poly-Fect transfection kit (QIAGEN), freshly cultured LM (tk^-) cells were transfected with plasmid DNA of pcDNA3.1-vhs, pgEp-vhs, pcDNA-3.1, or pCAT-Promoter (alone or in combination). After 48 h of transfection, cells were scraped and disrupted by freezing and thawing three times, and the CAT reaction was carried out with the method developed by Gorman et al. (1982). Following the reaction, the non-acetylated (CAM) and acetylated (Ac-CAM) forms of (^{14}C)-labeled chloramphenicol were separated by silica thin layer chromatography (TLC), and the silica sheet was exposed to X-ray film for autoradiography.

Plaque assay

For plaque assay, confluent monolayers of LM (tk^-) cells were either mock or were transfected with pgEp-vhs or pgEp-EGFP. At the 16 h after transfection, cells were infected with PRV by the multiplicities of infection (MOI) of 0.1 PFU/cell. The inoculum was removed after additional 2 h and cells were then overlaid with medium containing 1% methylcellulose. After 48 h of infection, the plaque formation was visualized by staining with 0.5% crystal violet.

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